Genotypic and phenotypic analyses of aac(3) aminoglycoside-resistance gene diversity point to three distinct phenotypes of contemporary clinical relevance

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Genotypic and phenotypic analyses of *aac(3)* aminoglycoside-resistance gene diversity point to three distinct phenotypes of contemporary clinical relevance

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Madam,

Whole-genome sequencing (WGS) and resistance gene annotation (RGA) has increasingly evolved as a routine method for molecular typing of clinical bacterial isolates in the diagnostic microbiology laboratory. WGS has proven particularly useful in outbreak detection and epidemiological surveillance and holds promise for in silico antimicrobial susceptibility testing (AST). However, a review by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has highlighted the need for additional evidence in support of WGS-inferred AST to guide clinical decision-making [1].

To unfold the full AST potential of WGS and RGA, a robust association between resistance genes and their effect on the phenotype needs to be established, taking into account an ever-evolving complexity of circulating gene variants. To underscore the significance, we studied the clinical prevalence and phenotypic diversity of the AAC(3) family of aminoglycoside acetyltransferases, a family of resistance genes conferring resistance to aminoglycoside antibiotics [2,3]. Despite considerable genetic diversity of *aac(3)* genes and 11 subtypes of AAC(3) enzymes previously described, our findings suggest a simplified scheme of four distinct clades, each with its own distinctive phenotype.

We analysed the resistance gene annotations of 239 772 human bacterial isolates deposited in the NCBI National Database of Antibiotic Resistant Organisms (NDARO) [4] to identify all genomes that code for at least one *aac(3)* gene. Subtype *aac(3)-I* was found to be the predominant *aac(3)* gene in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates, whereas *aac(3)-II* was the predominant subtype in *Enterobacterales* isolates (Fig. 1A). Subtypes IV and VI annotated more rarely and almost exclusively to *Enterobacterales* isolates. Subtypes III, V, and VII–XI were either absent or only of marginal presence in the NDARO annotations, suggesting the clinical relevance of these to be low (Supplementary Table S1). In summary, we consider only three subtypes to be of significant clinical relevance: I, II and, to a much lesser extent, IV. The presence of one or more *aac(3)* resistance genes was frequently found in Gram-negative clinical isolates; *aac(3)* genes were virtually absent from Gram-positive isolates and mycobacteria. A detailed description of the database analysis including all of the relevant *aac(3)* resistance gene variants listed in the NDARO Reference Gene Catalog is provided as Supplementary material.

Next we cloned the coding sequences of the clinically most prevalent (Supplementary Table S1) *aac(3)* amino acid sequences for each subtype into the *Escherichia coli* laboratory strain DH5α. Promoter control allowed defined expression of individual resistance genes in an otherwise isogenic background and without interference by other phenotypic determinants, as previously described [5]. The aminoglycoside susceptibility patterns of the engineered *aac(3)* strains in broth microdilution assays revealed four distinct phenotypes across the five subtypes studied (Fig. 1B). Subtype *aac(3)-I* conferred high-level resistance to gentamicin only. Subtypes II and VI conferred resistance to both gentamicin and tobramycin. Subtype *aac(3)-III* conferred resistance to gentamicin, tobramycin and neomycin, whereas subtype *aac(3)-IV* conferred resistance to gentamicin, tobramycin, neomycin, and apramycin. These four structurally distinct aminoglycosides appeared to provide comprehensive and representative differentiation, since testing of additional aminoglycoside antibiotics did not result in further differentiation (data not shown). Susceptibility to amikacin, for instance, another important aminoglycoside in clinical use, was not affected by *aac(3)* resistance genes.

Phylogenetic analysis of the amino acid sequences of the NDARO reference genes indicated genotypic clustering in accordance with the observed phenotypic resistance profiles (Fig. 1C). Subtype VI, for instance, which was found to share a susceptibility profile with subtype II, also showed phylogenetic proximity to AAC(3)-II, prompting us to define a common clade for the two subtypes. Annotation of an *aac(3)* gene was always indicative of gentamicin resistance. Tobramycin is often used in the treatment of *P. aeruginosa* infections, which is in agreement with retained activity against isolates that test positive for an *aac(3)* gene, because the large majority of these would be of subtype I, which does not appear to confer significant levels of resistance to tobramycin. In the case of *Enterobacterales* isolates, however, the annotation of an *aac(3)* gene is likely to be of subtype II, rendering the pathogen resistant to both gentamicin and tobramycin, while retaining aminoglycoside susceptibility to neomycin and apramycin.

In summary, our results not only highlight careful differentiation of resistance gene variants as a critical prerequisite for indicative RGA, but also demonstrate a path forward towards in silico AST by combining systematic data mining with surrogate phenotypic AST of distinct gene variants of clinical relevance.

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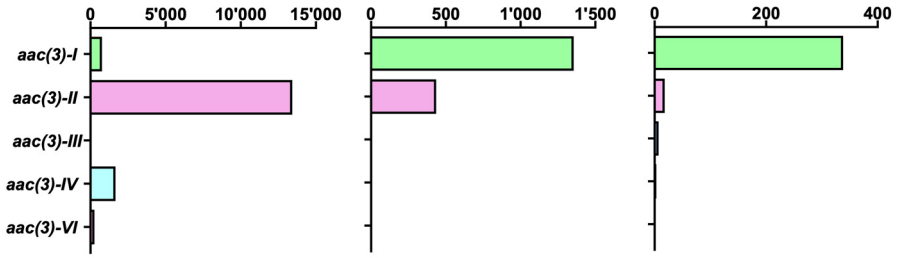
A)



B)

Genotype	GenBank accession no.	Fold increase in MIC relative to wild-type			
		GEN	TOB	NEO	APR
Wild-Type		1	1	1	1
AAC(3)-I					
aac(3)-Ia	WP_002089484	64-128	2	1	1
aac(3)-Ib	WP_010792467	32-64	2-4	1	1
aac(3)-Ic	WP_034006142	64-128	2	1	1
aac(3)-Id	WP_003155734	64-128	8	1	1
AAC(3)-II					
aac(3)-IIa	WP_063840264	>128	32-64	1	1
aac(3)-IIc	WP_063840266	>128	8-16	1	1
aac(3)-IId	WP_000557454	>128	64	1	1
aac(3)-IIe	WP_000557452	>128	64-128	1	1
aac(3)-IIg	WP_012695485	>128	16-32	1	1
AAC(3)-III					
aac(3)-IIIa	WP_063840261	>128	>128	32	1
AAC(3)-IV					
aac(3)-IV	WP_001199192	>128	>128	8-16	>128
AAC(3)-VI					
aac(3)-VI	WP_000131886	>128	32-64	1	1

C)

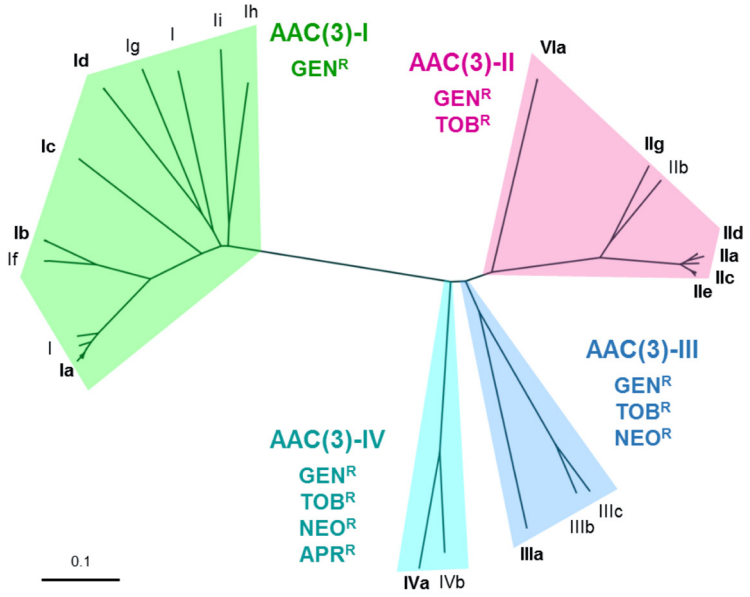


Fig. 1. Clinical prevalence, phenotypic drug resistance, and genotypic diversity of the AAC(3) family of aminoglycoside acetyltransferase genes. (A) Summary of *aac(3)* resistance gene annotations in 239 772 human bacterial isolates deposited in the NCBI National Database of Antibiotic Resistant Organisms (NDARO). (B) Phenotypic susceptibility of engineered *Escherichia coli* strains to the four chemically distinct aminoglycoside antibiotics gentamicin (GEN), tobramycin (TOB), neomycin (NEO), and apramycin (APR). Each *E. coli* strain heterologously expresses an individual *aac(3)* gene under defined promoter control, in an otherwise isogenic background. Phenotypic susceptibility is expressed as a fold increase in the minimum inhibitory concentration (MIC) relative to the parental wild-type strain. (C) Unrooted phylogenetic tree of the amino acid sequences of the 3-*N*-aminoglycoside acetyltransferase enzyme family AAC(3). Subtypes cloned and assessed in this study are highlighted in bold face. Phenotypic distinction associated with each clade is indicated by colour shading: GEN^R, gentamicin resistance; TOB^R, tobramycin resistance; NEO^R, neomycin resistance; APR^R, apramycin resistance. The NCBI accession numbers of the individual protein sequences are listed in Supplementary Table S3.

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Ethical approval: Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.11.012.

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